STUDIES ON THE TEMPERATURE DEPENDENCE OF CHROMATOGRAPHY ON A DEXTRAN GEL (SEPHADEX G-200)*

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INTRODUCTION

A number of papers have been published in recent years on the theoretical aspects of gel chromatography (gel filtration), a method which is used to separate molecules according to size[1–5]. Most authors favour a chromatographic mechanism based on the preferential steric exclusion of the larger molecules from the gel bed, *i.e.* an entropy effect. In one model, proposed by LAURENT AND KILLANDER[3], the chromatographic process was treated in terms of exclusion of spherical molecules from a random network of fibers. This treatment has proved quite useful in explaining quantitatively the gel chromatographic data from experiments on different gels[6–9].

ALBERTSSON has, however, pointed out that surface forces between the molecules and the surrounding polymer medium may give a similar effect since the magnitude of these forces should be proportional to the surface area of the molecule and thus its size[10].

A study of the temperature dependence of the chromatographic process should distinguish between these two hypotheses since only in the latter case should a dependence be found. The results of such a study are presented in this paper.

MATERIALS

The dextran gel Sephadex G-200 (Lot No. To-6471), a dextran fraction (FDR 922) with a mol. wt. of $2 \cdot 10^6$, and dextran (mol. wt. $4 \cdot 10^4$) substituted with fluorescein isothiocyanate ($0.15 \cdot 10^{-2}$ g fluorescein isothiocyanate bound to 1 g of dextran) were kindly supplied by Drs. B. GELOTTE, K. GRANATH and A. DE BELDER, AB Pharmacia, Uppsala, Sweden. Three homogeneous and well-characterized Ficoll fractions, No. XIII, XV, and XVI with the Stokes' radii 49 Å, 34.5 Å and 26.4 Å respectively, were available in our own laboratory[11]. Ficoll is a highly cross-linked polysucrose. The values of the Stokes' radii had been obtained by chromatography on a gel column calibrated with well known proteins[11]. Tritiated water was obtained from The Radiochemical Centre, Amersham, England.

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METHODS

Gel chromatography

Sephadex G-200, suspended in 0.2 *M* NaCl and heated in a boiling water bath for 30 min to facilitate rapid swelling, was packed at room temperature in a 95 × 2 cm tube similar to that described by PORATH AND BENNICH[12]. Experiments were performed alternately at 9° and 60°. The eluant 0.2 *M* NaCl, was deaereated and preheated before entering the column and all connecting tubing was covered with metal foil to prevent gas exchange. This effectively prevented the formation of air bubbles in the column at the higher temperature. The flow rate was kept at 19 ml/h with a pump. Fractions of 2.5 ml were collected and weighed.

The void volume and total volume of the column were repeatedly checked by chromatography of dextran FDR 922 and ^3H_2O. The Ficoll fractions XIII, XV and XVI were chromatographed separately twice at each temperature. 5 mg samples of the polysaccharides and 0.1 μCi of ^3H_2O were applied on the column in 1 ml 0.2 *M* NaCl. The anthrone method[13] was used to localize the dextran and Ficoll peaks. ^3H_2O was analyzed in a Packard Tri-Carb liquid scintillation counter.

Fluorescence polarization

The polarization of fluorescence was measured in a Zeiss spectrofluorometer equipped with two quartz prism monochromators (type M4Q II) and a xenon arc lamp (XBO 501) as the exciting source. The exciting radiation was polarized by quartz Polacoat Polarizing filters* and the emitted light analyzed by Polaroid filters**. The emission was observed at 90° to the exciting light beam and the light components vibrating parallel $I_{\parallel}$ and perpendicular $I_{\perp}$ to the exciting light beam were determined. To correct for polarizing effects of the optical system, both components of the emitted light were determined with the plane of polarization of the exciting light turned 90° from its original position; in this way both vectors of the emitted light are positioned at right angles to the vector of the polarized light. The amplitudes of both emission vectors thereby attain in theory the same magnitude[14] and from their ratio the correction factor for the superimposed polarization of the optical system can be determined[15]. The fluorescence was excited at 480 nm and 26 nm bandwidth and observed at 530 nm and 8.5 nm bandwidth.

Determinations were made on solutions of fluoresceinylthiocarbamoyl-dextran which were 10^{-5} *M* with respect to the fluorescein concentration. The medium used was 0.1 *M* sodium chloride and 0.001 *M* phosphate buffer, pH 6.8. The viscosity was varied by the addition of sucrose in one series of experiments, carried out at room temperature. In another series the temperature was varied between 10° and 75°, and no sucrose was added.

RESULTS

The chromatograms obtained with the different materials at 9° and 60° are shown in Figs. 1a and b, and the elution volumes are tabulated in Table I. The void volume, V_0, was taken as the elution volume of dextran FDR 922. The total volume,

* Polacoat Inc., Blue Ash 42, Ohio.
** Polymer Research Div., Polaroid Corp., Cambridge, Mass.

V_t, was determined from the elution volume of ^3H_2O to which a small correction was added corresponding to the volume occupied by the dextran chains adjusted to account for tritium exchange between the water and the polysaccharide[16]. The elution volume for Ficoll is denoted by V_e. Chromatographic runs were performed alternately at 9° and 60° with high reproducibility.

The partition coefficients, K_{av}, between the gel phase and liquid phase for the Ficoll fractions are listed in Table II. They were obtained with the following equation[3]:

$$K_{av} = \frac{V_e - V_0}{V_t - V_0} \qquad (1)$$

Fig. 1 and Table I show that the total volume of the gel phase given as $V_t - V_0$ is smaller at 60° than at 9° indicating a shrinking of the gel at the higher temperature.

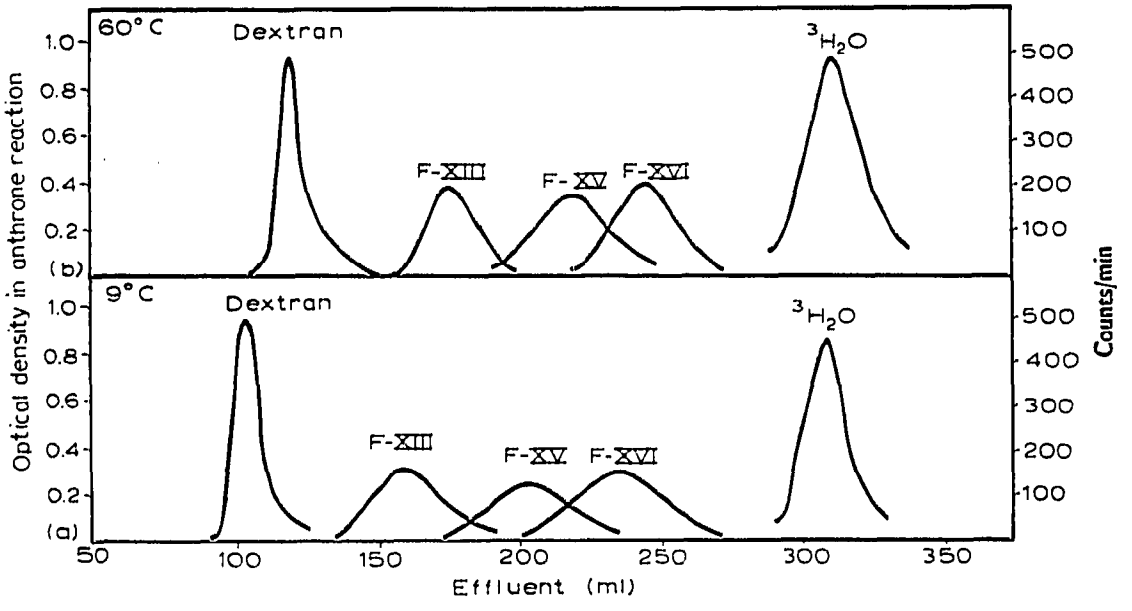

Fig. 1. Chromatography of dextran FDR 922, three Ficoll fractions and ^3H_2O at (a) 9° and (b) 60° on a 95 × 2 cm column of Sephadex G-200.

There are also slight differences in the K_{av}-values at the different temperatures (Table II).

The shrinking indicated a structural change in the dextran gel, supposedly due to a change in the polysaccharide–solvent interaction. To verify the structural change with temperature, fluorescence polarization measurements were performed on

TABLE I

CHROMATOGRAPHIC DATA FOR THE DIFFERENT RUNS

The volumes are expressed in ml.

	V_0	V_t	V_e (*F-XIII*)	V_e (*F-XV*)	V_e (*F-XVI*)	$V_t - V_0$
9°	105	314	160	205	236	209
60°	121	317	177	220	246	196

fluoresceinylthiocarbamoyl-dextran. The experimental data are plotted in Fig. 2 as suggested by WEBER[17–19]. In this figure, $1/p-1/3$ is plotted against T/η, where p is the polarization of the fluorescence, T the absolute temperature and η the viscosity of the medium in poise. For changes in viscosity at constant temperature, the plot gives a straight line. When the temperature was varied and water was the solvent, the plot against T/η afforded an exponential curve intercepting the straight line at a value of T/η corresponding to 20° and the viscosity of water.

TABLE II

PARTITION COEFFICIENTS FOR THE DIFFERENT FICOLL FRACTIONS

Ficoll	K_{av} *observed at 9°* (*A*)	*Calculated value of* K_{av} *at 60° from eqn. (4) and value at 9°* (*B*)	K_{av} *observed at 60°* (*C*)	(*B*–*A*)	(*C*–*A*)
XIII	0.263	0.323	0.286	0.060	0.023
XV	0.479	0.536	0.505	0.057	0.026
XVI	0.627	0.674	0.638	0.047	0.011

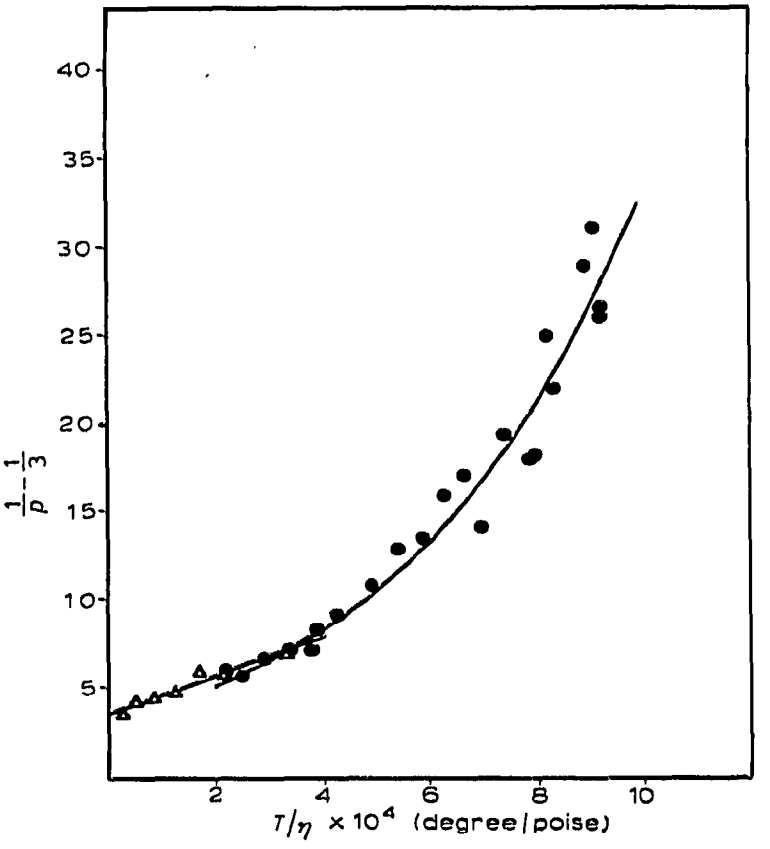

Fig. 2. Plot of fluorescence polarization data obtained on fluoresceinylthiocarbamoyl-dextran. (△) Values obtained at constant temperature in sucrose solutions of varying concentrations. (●) Values obtained at varying temperature in buffer. For details see text.

DISCUSSION

The present work was initiated in order to determine whether the gel chromatographic mechanism is based on entropy effects or surface forces between the gel matrix and the molecules.

Sterix exclusion of spherical molecules from a random network of straight fibers is described by the following equation[3,20]:

$$K_{av} = \exp(-\pi L(r_s + r_f)^2) \quad (2)$$

where K_{av} is the fraction available in the network for a molecule with the radius r_s, L is the total length of the fibers per unit volume and r_f is the radius of the fibers.

If $r_s \gg r_f$ and since $4\pi r_s^2$ is the surface area, A, of the spherical molecule, the equation becomes:

$$K_{av} = \exp\left(-\frac{AL}{4}\right) \quad (3)$$

K_{av} for a molecule in a gel network can be determined experimentally and is equal to the partition coefficient between the stationary gel phase and the mobile liquid phase in gel chromatography.

ALBERTSSON[10], on the assumption that surface forces are responsible for the partition of a molecule between two polymer phases derived the following expression for the partition coefficient between the two phases (eqn. (18) ref. 10, p. 100).

$$K = \exp\left(\frac{A\lambda}{kT}\right) \quad (4)$$

were λ is a parameter determined by the nature of the surface forces, k is Boltzmann's constant and T the absolute temperature.

An essential difference between eqns. (3) and (4) is the temperature parameter in the latter case.

In Table II, the K_{av}-values at 60° calculated according to eqn. (4) using the corresponding values at 9° are compared with the experimentally observed values. The observed increase in K_{av} at 60° was on average a third of that expected from eqn. (4).

Although the agreement was poor there is a definite change in K_{av} with temperature in the direction expected. Therefore a contribution to the gel chromatographic mechanism from surface forces cannot be excluded, although a more likely explanation is the following.

When the temperature is increased, a marked shrinking of the gel is observed, corresponding to a 6 % decrease of its volume between 9° and 60°. This must be due to a change in polysaccharide–solvent interaction, probably leading to an increased flexibility in the polymer chain at higher temperatures. An increased flexibility will result in a more pronounced coiling of the polymer chain, which will affect its exclusion properties. Fluorescence polarization measurements were therefore performed in order to demonstrate the increased flexibility in the dextran molecule with temperature.

PERRIN[21,22] and later WEBER[17,19] have shown that the degree of polarization

of the fluorescence emitted from a spherical macromolecule is related to the relaxation time of the macromolecule according to the equation:

$$\frac{1}{p} - \frac{1}{3} = \left(\frac{1}{p_0} - \frac{1}{3}\right)\left(1 + \frac{3\tau}{\rho}\right) \qquad (5)$$

where ρ is the relaxation time and τ is the lifetime of the excited state. ρ is a linear function of $(\eta/T)V$, where η is the viscosity of the medium, T the absolute temperature and V the molecular volume of the macromolecule in solution. p_0 is the limiting polarization when $T/\eta \rightarrow 0$. This equation (5) applies also to ellipsoidal macromolecules where ρ is substituted by ρ_n, the harmonic mean of the two principal relaxation times of the rotation of the ellipsoidal molecule.

Fig. 2 shows that when $1/p - 1/3$ is plotted against T/η and the viscosity is varied by the addition of sucrose at constant temperature, a linear relationship is obtained. On the other hand, when T/η is varied by change in the temperature, a non-linear function convex towards the T/η axis is obtained which intercepts the line at a value of T/η corresponding, as expected, to 20° and the viscosity of water. The temperature rise thus seems to induce a change in the relaxation times of the dextran chains, which cannot be ascribed to their ordinary changes in Brownian motion with temperature. In the latter case the points from the two experiments should have formed a continuous function.

Furthermore, according to WEBER[19] a curvature convex towards the T/η-axis can be present for any type of macromolecules only if the relaxation time is not a linear function of η/T. Of the various explanations given by WEBER to the non-linear relaxation time, the only one which seems applicable to the dextran molecule is the appearance at higher temperatures of new rotational degrees of freedom that were frozen at lower temperatures. That the relaxation time decreases more than expected with increasing temperature is thus best interpreted as an increased flexibility in the polymer chains, presumably due to a change in the polysaccharide–solvent interaction.

It is improbable that the results are due to an increased lifetime of the excited state in the temperature interval observed, as no such effect is observed in free fluorescein[23,24]. The results cannot be due to a scission of the covalent bond between fluorescein and dextran at high temperature, as the temperature effect is reversible.

An increased flexibility of the dextran chains will result in an increased coiling *i.e.* the dextran chains will be shorter and thicker. The gel chromatography data can be used to test this quantitatively. Eqn. (2) includes the parameters L and r_r which denote the length of the dextran chains per unit volume and their radius respectively. SIEGEL AND MONTY[25] have proposed that eqn. (2) should be written (see also ref. 8):

$$(-\ln K_{av})^{\frac{1}{2}} = (\pi L)^{\frac{1}{2}}(r_r + r_s) \qquad (6)$$

In Fig. 3 $(-\ln K_{av})^{\frac{1}{2}}$-values for the different Ficoll fractions are plotted against their Stokes' radii (r_s). The points at 9° and 60°, respectively, fall on straight lines as eqn. (6) demands. The intercept on the abscissa indicates that the radius of the dextran chain at 9°, is 6 Å and at 60°, 8 Å. The parameter L, obtained from the slope of the lines is at 9°, $15 \cdot 10^{11}$ cm/ml and at 60°, $13 \cdot 10^{11}$ cm/ml.

The gel chromatography data are thus in very good agreement with the assumption that the dextran chains in the gel are more flexible at higher temperatures

leading to increased coiling and shorter and thicker fibers. This seems to be the most reasonable explanation of the temperature dependence observed during the gel chromatography on Sephadex G-200. It should further more be pointed out that the results of the plot in Fig. 3 are not compatible with eqn. 4 since the latter predicts that the lines should, independent of temperature, pass through the origin.

It is improbable that the temperature would affect the structure of the Ficoll fractions, used in the experiments, since this substance is highly crosslinked and thus very rigid.

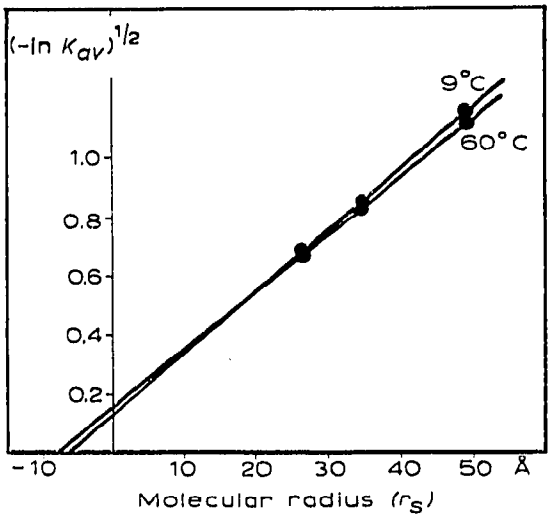

Fig. 3. Test of the structure of the dextran gel at different temperatures with use of the gel chromatography data. For details see text.

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SUMMARY

Three Ficoll fractions have been chromatographed on a dextran gel (Sephadex G-200) at 9° and 60°. The results indicate that the separation of molecules according to their size in this kind of chromatography is largely due to an entropy effect (steric exclusion).

A certain temperature dependence was, however, observed. This can probably be ascribed to a structural change in the dextran gel, resulting in a shrinking of the gel at higher temperatures. Fluorescence polarization measurements on fluoresceinyl-thiocarbamoyl-dextran confirmed that an increased flexibility in the dextran chains occurred at higher temperature.

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